

**TUMOR SUPPRESSOR GENE EXPRESSION IN CANINE  
OSTEOSARCOMA: AN IMMUNOHISTOCHEMICAL STUDY**

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## **Hypothesis and Objectives**

The overall goal of this proposal is to determine expression of the tumor suppressor genes PTEN, p16, Rb, and p53 in canine osteosarcoma using immunohistochemistry.

*Hypothesis: Loss of tumor suppressor genes PTEN, p16, Rb, and p53 protein expression occurs frequently in canine osteosarcoma.*

**Objective 1: Identify cases of canine osteosarcoma presented to The Ohio State College of Veterinary Medicine Veterinary Medical Center between the years 2003-2008 and abstract the associated clinical information from medical records.**

A medical record database including each affected patient's related clinical information will be constructed. Relevant patient information includes, but is not limited to, age, breed, sex, date of diagnosis, date of death, cause of death, and treatment protocol used.

**Objective 2: A tissue microarray (TMA) containing each dog's osteosarcoma tumor biopsy will be constructed from formalin-fixed paraffin embedded (FFPE) blocks.**

TMAs will be constructed from individual patient FFPE tissue blocks. TMAs will be constructed with the assistance of the Immunohistochemistry Core in the College of Veterinary Medicine.

**Objective 3: Determine protein expression of selected tumor suppressor genes using immunohistochemistry (IHC).**

IHC will be performed on canine osteosarcoma TMAs for selected tumor suppressor proteins (PTEN, Rb, p16, and p53). The incidence of loss of tumor suppressor protein expression in canine osteosarcoma tumor samples as determined by IHC will be determined.

**Significance and Justification**

In order to optimize care for human and canine patients affected with cancer, it is important to identify dysregulated cancer associated genes that might be used as prognostic indicators or could be used to help develop more effective treatments. Since the introduction of adjuvant chemotherapy to treat potential micrometastatic disease and subsequent modifications of adjuvant cytotoxic chemotherapy protocols, no significant advances in the treatment of canine osteosarcoma have been made for the past 20 years.<sup>[15]</sup> Because the biologic behavior, including metastatic potential, depends significantly on the underlying genetics of the tumor, including the expression of tumor suppressor genes, it is important to obtain a better understanding of the incidence of tumor suppressor gene dysregulation in osteosarcoma in dogs. Importantly, the signaling pathways associated with tumor suppressor genes are potential targets for therapy and their expression may be correlated with clinical outcomes.

By determining which tumor suppressor proteins are being aberrantly expressed, future therapies can be targeted to that signaling pathway. For example, mutations affecting the pRb pathway make cells refractory to antigrowth factors that normally inhibit cells from entering the

G1 phase of the cell cycle.<sup>[5]</sup> Thus, restoring Rb function might be one form of anticancer treatment.

In conclusion, studying signaling pathways and gene families linked to cancer at the molecular level has revealed that optimal treatment methods for different types of cancer are not universal. There are many different genes linked to cell proliferation and cell growth inhibition, and an abnormality in any one of these genes could result in uncontrolled cell growth. Therefore, based on the actions of the genes involved, responses to different treatments in different patients are expected to vary. By understanding which cancer genes are dysregulated in a particular tumor, the prognostic significance of that dysregulation can be determined and treatment protocols can be established so that they are personalized to each patient. In addition, identifying these prognostic indicators will then identify more targets for future studies.

## **Background**

### **Canine and Pediatric Osteosarcoma**

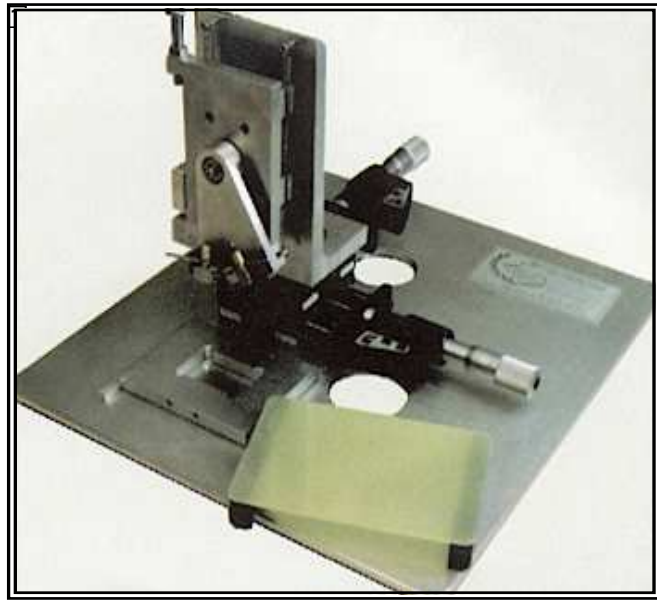
Osteosarcoma (OS) is a heterogeneous tumor of mesenchymal origin accounting for approximately 80% of bone tumors arising in dogs. While any dog can be afflicted with OS, large breed dogs between the ages of 7-10 years are most commonly affected.<sup>[16]</sup> The most frequent anatomic site for OS occurrence is in the appendicular skeleton, but it can also occur in the vertebral column, skull, and pelvic bones of the axial skeleton. Although amputation, chemotherapy, and radiation therapy are available treatment options, the long-term prognosis is poor. Because the onset of OS is sudden, many diagnoses are not made until the tumor has

already metastasized <sup>[13]</sup>. The lungs are the most frequent site of metastasis; however, other soft tissue and bone metastases are also possible. Dogs that undergo amputation and respond well to chemotherapy have an estimated median survival time of about one year. Survival times are significantly lower without amputation or treatment. A patient who receives no treatment generally has a 1-3 month survival time. <sup>[16]</sup>

A significant breakthrough in the field of comparative oncology came with the release of the canine genome in 2005 and subsequent application of genomic methods to understanding canine tumor biology. <sup>[17]</sup> It is now known that cancer related genes and pathways associated with cancer in dogs are similar to those associated with cancer in humans. Consequently, spontaneously occurring cancer in pet dogs is a highly relevant model for studying similar cancers in people. While OS in dogs is more prevalent among older dogs, human OS primarily affects the pediatric age group at a rate of 8.7 children per million. <sup>[2]</sup> Human OS often occurs in the metaphyseal regions of the long bones (same as dogs), and generally occurs in adolescence. <sup>[2]</sup> Although the initiation of multidrug chemotherapy and more efficient surgical methods in the 1980s increased the rate of human nonmetastatic OS to 65%, OS treatment in humans since that time has not improved. <sup>[2]</sup> Because canine OS is similar to human OS in regards to its metastatic and aggressive nature, histology, mode of treatment, similarity in treatment response, and progression, dogs are an excellent model for comparative study.

## Tissue Arrays

An efficient method for analyzing and comparing a large number of tissue samples in a high throughput fashion is to construct a tissue array. A tissue array is constructed by using a high precision instrument to punch a small portion of tumor tissue preserved in a formalin fixed paraffin embedded block (FFPE) and then inserted it into a recipient paraffin block. Because a large number of tumor samples can be

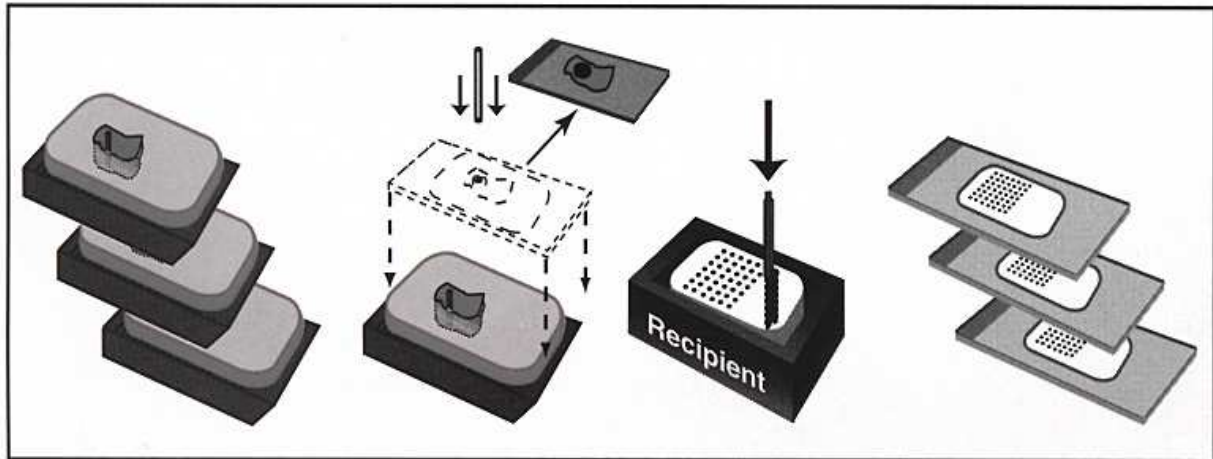


**Figure 1.** A high precision instrument is used to punch a sample of fixed tissue and transfer it to a recipient block. A slide is then made from the block, allowing the samples from different patients to be tested simultaneously on a single slide.

inserted into a single recipient paraffin block, constructing a tissue array allows numerous tumor biopsies to be assayed simultaneously, thus saving time, cost and ensuring consistent treatment methods among all samples.

In order to construct a tissue array, slides containing the tumor biopsy must be reviewed to confirm the diagnosis and to mark the tumor location on the slide. Then, the slide is matched to its original block to locate the proper location for “punching” (sampling) the original block. The punching instrument can remove samples as small as 0.6 mm and as large as 2.0 mm. The arraying device deposits precise amounts, “cores”, of each sample into premade holes in the recipient block (Figure 1 and Figure 2). The completed tissue array can then be used for essentially any application for which individual FFPE tissue sections are normally used, e.g.

immunohistochemistry, in situ hybridization, fluorescence in situ hybridization (FISH); however, the TMA allows up to hundreds of samples from different patients can be evaluated on a single slide.



**Figure 2. TMA construction.** 1. Tissue preserved in formalin fixed paraffin-embedded donor blocks. 2. A manual high-precision instrument removes a cylindrical core from representative tissue in the donor block. 3. The cores are inserted into a recipient paraffin block. 4. Slides are constructed for analysis by cutting 0.5µm sections from the TMA block and staining with H&E.

### **Tumor Suppressor Genes Implicated in Canine OS Pathogenesis**

Tumor suppressor genes are genes that protect a cell from uncontrolled cell division deregulated.

When deregulated or mutated to cause a loss in gene function, the cell can progress to cancer.

Tumor suppressor proteins typically have a repressive effect on the cell cycle or promote apoptosis. Several tumor suppressor genes have been identified previously to be dysregulated in canine OS; however, the overall prevalence of tumor suppressor gene dysregulation and its prognostic significance in canine osteosarcoma has not been evaluated.

## **p53**

The tumor suppressor gene p53 has been called the “policeman” of the cell cycle, or “guardian of the genome”. If chromosomal DNA becomes damaged, p53 halts the cell division process until repair can be made, or directs the cell towards apoptosis if the damage is irreparable. When the p53 gene is damaged or mutated, cells can replicate uncontrollably. The locations and types of mutations seen in the p53 gene in dogs are nearly identical to those seen in the human p53 gene.

<sup>[10]</sup> Although p53 can be inactivated by mutations such as insertions or deletions, point mutations are the most common mutation seen in human OS. Past studies have shown that p53 is inactivated in approximately 50% of human cancers and 60% of canine cancers. <sup>[7][10]</sup> In dogs, p53 is over-expressed in 84% of OS from appendicular locations and 54% of OS from axial locations. <sup>[7]</sup> Normally, the p53 protein is not stable enough to be seen on IHC. When the p53 becomes mutated, the protein becomes abnormally stable and IHC can readily detect p53 in the cell’s cytoplasm. <sup>[6]</sup>

## **Rb**

The retinoblastoma susceptibility gene codes for the retinoblastoma protein (Rb) which regulates passage from G1 to S phase of the cell cycle. Rb regulates cell growth by regulating the amount of available transcription factors from the E2F family. <sup>[6][7]</sup> Cyclin-dependent kinases (CDKs) block Rb phosphorylation when cell growth and DNA synthesis need to be arrested. A mutation that causes inhibition of CDKs or Rb phosphorylation allows for unregulated cell growth, and occurs in many human cancers. Although Rb originates in the cytoplasm of the cell, it carries



out its function in the nucleus. Decreased, or absent, expression of the Rb protein has been reported in canine OS cell lines.<sup>[7][8]</sup>

## **PTEN**

Another tumor suppressor gene whose dysregulation contributes to the development of cancer is PTEN. By antagonizing the phosphoinositide-3 kinase pathways (PI3K), PTEN negatively impacts cell survival signals and growth, and may have inhibitory effects on metastases from tumor cell invasion and tumor directed angiogenesis.<sup>[6][8]</sup> When PTEN is mutated, the PI3K pathways are no longer inhibited, promoting cell survival and proliferation. Three of 5 canine OS cell lines and 10 of 15 canine OS tumors exhibited decreased or absent PTEN expression in one study.<sup>[8]</sup>

## **P16**

The tumor suppressor gene p16 functions as a CDK inhibitor that prevents cell cycle progression and directs cells toward apoptosis.<sup>[6]</sup> When p16 protein expression was evaluated using immunocytochemistry in canine melanoma, staining was primarily cytoplasmic and reduced expression was seen in 21 of 26 melanoma samples.<sup>[6]</sup> When evaluated in canine OS, p16 expression was not detectable in 4 of 5 canine OS cell lines.<sup>[7]</sup>

**Table1. Tumor Suppressor Genes in Canine Osteosarcoma**

| Gene | Function   | Significance   | References |
|------|--|--|------------|
| Rb   | Regulator of cell growth between G1/S phases of cell cycle                           | Inhibition of CDKs or Rb phosphorylation allows for unregulated cell growth. In humans, one thousand times greater risk to develop OS if Rb is hereditary. | [2]        |
| P53  | Promotes apoptosis and policeman of cell cycle progression                           | Mutation is correlated with unregulated cell growth and a poor outcome. Inactivated in 50% human cancers and 60% of canine cancers.                        | [2][3]     |
| P16  | Prevents cell cycle progression past the G1 phase and directs cell towards apoptosis | Mutation causes unregulated cell growth. Family gene INK4A is deleted in 10% of patients.  | [6][2]     |
| PTEN | Antagonizes the phosphoinositide-3 kinase pathways (pI3k)                            | Mutation increases chance of invasion, increases tumor cell survival, and tumor directed angiogenesis.   | [6][8]     |

## **Materials and Methods**

### **Case selection and tissue array**

Cases were identified by a computer-based search of The Ohio State University College of Veterinary Medicine Pathology Archives for diagnoses of OS from the years 2003-2008. Two-hundred twenty-one cases were selected and reviewed by a board certified veterinary pathologist. Of the 221 cases reviewed, material was available for use from 165 of them. Patient

information including date of diagnosis and biopsy, breed, sex, cause of death, treatment method, survival time, disease-free interval, and age were recorded in a spreadsheet. Missing or unknown follow-up information was provided by the referring veterinarian.

Prior to constructing the tissue array, hematoxylin and eosin (H&E) stained slides were reviewed by a board certified pathologist who confirmed the diagnosis and type of OS, and marked the target area for coring that included a representative area of tumor. The target area on the slide was matched to the FFPE donor block and three 1.5 mm punches of tumor were removed and inserted into a recipient block.

### **Immunohistochemistry**

Slides are deparaffinated in xylene, 100% ethanol, 95% ethanol, 70% ethanol, and water. Slides were then incubated in Target Retrieval solution (Dako) at a 1:10 dilution in a decloaking chamber. Slides were then heated at 125°C for 30 seconds, and then cooled to 90°C for 10 seconds. After the slides were cooled at room temperature for 10 minutes, they were placed in 3% H<sub>2</sub>O<sub>2</sub> for 10 minutes to quench endogenous peroxidase activity.<sup>[10]</sup> To prevent nonspecific antibody bonding, the slides were then incubated with 3% nonfat dry milk (NFD) mixed with 150 mL phosphate buffered saline (PBS) for 30 minutes. Control slides were incubated with an irrelevant, isotype matched negative control. For PTEN detection, a monoclonal mouse anti-human antibody (Santa Cruz Biotechnology, INC) was used. Sections were incubated at a 1:100 dilution for 2 hours at room temperature. For detection of p16, a polyclonal rabbit anti-human antibody (Santa Cruz Biotechnology, INC) was used. Sections were incubated at a 1:100 dilution for 2 hours. A mouse monoclonal anti-human retinoblastoma protein antibody (BD

Pharminogen<sup>TM</sup>) was diluted 1:100 and incubated for 2 hours for Rb detection. For p53 detection, the polyclonal rabbit anti-p53 antibody was diluted 1:100 and incubated for 2 hours. After the primary antibody incubation period, slides were transferred to PBS and then incubated with the secondary antibody for 30 minutes (VectaStain® Universal Elite ABC Kit, Vector Laboratories). Slides were then transferred to PBS and then incubated with Reagent A and Reagent B (VectaStain® Universal Elite ABC Kit, Vector Laboratories) for 30 minutes. The sections were then incubated with DAB tablets (Sigma, St. Louis) prepared in tap water for 5 minutes, then counterstained with hematoxylin, dehydrated, and cover-slipped.

### Immunohistochemistry Scoring

Immunohistochemical staining was scored semi-quantitatively the following way:

**Table 2: Semi-quantitive Immunohistochemical Scoring**

| <b>Staining</b>          | <b>Distribution</b>               |
|--------------------------|-----------------------------------|
| Negative (<10% cells):0  | No staining: 0                    |
| Positive (>10% cells): 1 | Nuclear: 1                        |
|                          | Cytoplasmic: 2                    |
| <b>Intensity</b>         | Nucleolar: 3                      |
| Weak: 1                  | Cytoplasmic and nucleolar: 4      |
| Moderate: 2              | Cytoplasmic and nuclear: 5        |
| Intense: 3               |                                   |
|                          | <b>Cannot be interpreted: CBI</b> |

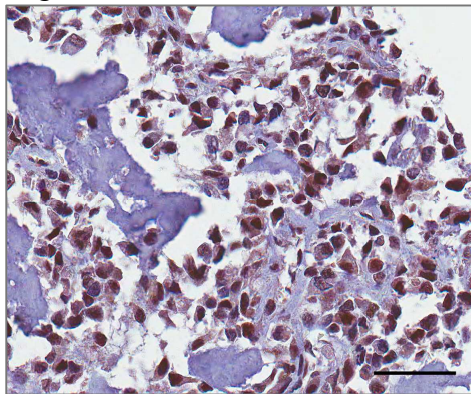
## Results

**Table 3: Immunohistochemistry Analysis of TS genes PTEN, p16, Rb, and p53**

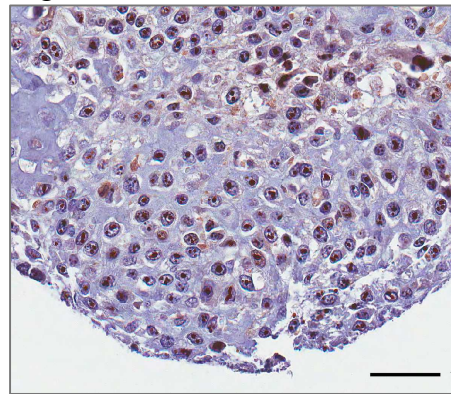
| Gene                      | PTEN |      | p16 |      | Rb  |      | p53 |      |
|---------------------------|------|------|-----|------|-----|------|-----|------|
|                           | N=   | %    | N=  | %    | N=  | %    | N=  | %    |
| <b>Samples</b>            | 147  |      | 149 |      | 146 |      | 151 |      |
| <b>Staining</b>           |      |      |     |      |     |      |     |      |
| Positive                  | 92   | 62.6 | 100 | 67.1 | 100 | 68.5 | 125 | 82.8 |
| Negative                  | 55   | 37.4 | 49  | 32.9 | 46  | 31.5 | 26  | 17.2 |
| <b>Distribution</b>       |      |      |     |      |     |      |     |      |
| Nucleolar                 | 20   | 13.6 | 0   | 0    | 0   | 0    | 0   | 0    |
| Cytoplasmic and Nuclear   | 26   | 17.7 | 35  | 23.5 | 81  | 55.5 | 98  | 65   |
| Cytoplasmic               | 24   | 16.3 | 65  | 43.6 | 8   | 5.5  | 25  | 16.6 |
| Nuclear                   | 1    | 0.7  | 0   | 0    | 11  | 7.5  | 2   | 1.3  |
| Cytoplasmic and Nucleolar | 21   | 14.3 | 0   | 0    | 0   | 0    | 0   | 0    |
| <b>Intensity</b>          |      |      |     |      |     |      |     |      |
| Weak                      | 33   | 22.4 | 20  | 13.4 | 31  | 21.2 | 35  | 23.2 |
| Moderate                  | 35   | 23.8 | 21  | 14.1 | 36  | 24.7 | 32  | 21.2 |
| Intense                   | 24   | 16.3 | 59  | 40   | 33  | 22.6 | 58  | 38.4 |

Out of 146 samples that were evaluated for Rb, 100 (68.5%, Figure 3a) stained positive. The samples stained primarily cytoplasmic and nuclear (55.5%) and were mostly moderate in intensity (24.7%). Of the 147 samples that were evaluated for PTEN, 92 (62.6 %) stained positive. The primary staining pattern was cytoplasmic and nuclear at 17.7% (Figure 3b), followed closely behind by cytoplasmic (16.3 %) and cytoplasmic and nucleolar (14.3 %) and was moderate in intensity at 23.8%.

**Figure 3a**



**Figure 3b**



**Figure 3ab.** Figure 3a shows Rb intense nuclear/cytoplasmic staining. Figure 3b shows PTEN intense nuclear/nucleolar staining.

Of the 151 samples that were evaluated for p53, 125 (82.8%) stained positive. The primary staining pattern was cytoplasmic and nuclear (65%) and most were intense in staining (38.4%). Of the 149 samples evaluated for p16, 100 (67.1%) stained positive. The location of stain was primarily in the cytoplasm (65%) and the intensity score was intense (40%).

## **Discussion**

Other studies suggest that tumor suppressor genes are dysregulated in canine osteosarcoma.

However, due to the limited sample size of these studies, the frequency of dysregulation remained unknown. Usually, loss of expression of tumor suppressor genes is thought to promote cancer formation. P53 is an exception as positive expression tends to be more indicative of gene dysregulation and the promotion of cancer. A previous study looking at PTEN expression found that 10 of 15 (66.7%) OS tumors showed variable or negative PTEN staining<sup>[8]</sup>. The results of our findings showed negative staining in 55 of 147 samples (37.4%). Also, p16 was lost in 4 of 5 (80%) of cases according to one study<sup>[7]</sup>. The results of our study found p16 loss in 49 of 149 (32.9%) cases. Loss of Rb expression has been showed in 70% of sporadic OS<sup>[2]</sup>. Our findings showed negative Rb expression in 46 of 146 cases (31.5%). Another study reviewing p53 expression found negative p53 expression in 44 of 125 (35.2%) OS tumors<sup>[10]</sup>. The results of our study showed negative p53 expression in 26 of 151 (17.2%).

## **Conclusion**

Tumor suppressor genes found to be commonly dysregulated in human osteosarcoma have also shown to be aberrantly expressed in canine osteosarcoma. Altered expression of these tumor suppressor genes has potential prognostic and therapeutic significance for disease and treatment of dogs with osteosarcoma.

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